# Interaction Between Sample Preparation Techniques and Colorimetric Reagents in Nitrite Analysis in Meat

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**The amount of nitrite measured in model and meat systems was a function of the interactions of the chloride and ascorbate concentrations with the method of sample preparation and the combination of Griess reagents used for colorimetric determination. Ascorbate caused loss of nitrite in the samples when heated and interfered in the Griess reaction, increasing the concentration of pigment formed from any given concentration of nitrite if sulfanilic acid and *N*-(1-naphthyl)-ethylenediamine were used, and decreasing the amount if sulfanilamide and 1-naphthylamine were used. The interference was eliminated by both the AOAC procedure and mercuric chloride addition, although the former were less effective at higher ascorbate concentrations. Chloride increased the amount of pigment formed from a given amount of nitrite with sulfanilic acid but had no effect on the amount of sulfanilamide pigment.**

Although sample preparation for the analysis of nitrite in meat products varies with respect to degree of dilution, temperature of heating, alkalinization, or addition of various precipitating agents, the most common method of analysis is the spectrophotometric measurement of a diazo pigment formed from some combination of Griess reagents (1–8). The amount of pigment formed is affected by the presence of ascorbate (1, 2) and chloride (3). Ascorbate reduces the formation of carcinogenic nitrosamines in cured meat products (9) but the addition of large amounts of ascorbate interferes in the Griess reaction. Low yields of pigment, therefore, could be due either to a real loss of nitrite in the meats or to the ascorbate interference. Until now there has been no way of distinguishing these 2 effects, but it has been reported recently that ascorbate will either enhance or decrease the amount of pigment formed, depending on the combination of Griess reagents used (10).

The purpose of this study was to determine if this differential effect can be used to detect residual ascorbate in cured meats and to determine the effectiveness of sample preparation procedures on removal of ascorbate. We used the specific combinations of sulfanilic acid (SAA) with *N*-(1-naphthyl)-ethylenediamine (NED) and sulfanilamide (SAN) with 1-naphthylamine (1-NA), because these 2 combinations showed the greatest differences between the amount of pigment produced from any given amount of nitrite in the presence of ascorbate. For brevity, we shall refer to the first combination as SAA, and the second as SAN.

## Experimental

### *Model System*

Four model stock solutions were prepared in ionic strength $\Gamma/2 = 0.03$ acetate buffer, pH 5.5, each containing 2 mM nitrite and either 0, 3, 6, or 12 mM ascorbate. Half of each solution was made 1M in NaCl by addition of the appropriate amount of solid salt. All solutions were analyzed for nitrite, and then heated 1 h at 70°C in air. We chose pH 5.5 as the lower end of the range of meat pH values, to ensure an appreciable nitrite loss.

### *Meat System*

A slurry was prepared by blending pork semitendinosus muscle with an equal volume (w/v) of 6 mM sodium nitrite. Aliquots of the slurry were combined with half their volumes of solutions containing 0, 9, 18, or 36 mM sodium ascorbate, respectively. Each of these slurries was then divided into 3 aliquots and NaCl was added so that samples at every level of sodium ascorbate contained 0, 0.5, or 1.0M NaCl. Half (10 mL) of each of the 12 samples was placed in a controlled water bath 1 h at 70°C. All of the 24 (12 raw and 12 cooked) samples were analyzed for nitrite as soon as prepared. The pH was 5.62.

### *Nitrite Analyses*

*Direct analysis.*—Aliquots of the slurries were centrifuged 20 min at 48 200 g. The clear supernates were carefully removed with a syringe fitted with a 28 gauge needle. One-tenth mL was added to 1.0 mL of each appropriate Griess reagent solution in a 10 mL volumetric flask and diluted to volume. Turbidities of the pigment solutions were determined at 600 nm but were not of practical significance.

*Mercuric chloride addition.*—A saturated mer-

**Table 1. Effects of NaCl, ascorbate, sample preparation methods, heat, and Griess reagent on measured nitrite (mM $NO_2^-$) in model systems [a] (pH 5.5; [$NO_2^-$] initial = 2.0 mM)**

| | | Unheated | | | | | | Heated 70°C, 1 h | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Direct | | $HgCl_2$ | | AOAC | | Direct | | $HgCl_2$ | | AOAC | |
| M NaCl | mM Asc. | SAA | SAN | SAA | SAN | SAA | SAN | SAA | SAN | SAA | SAN | SAA | SAN |
| 0 | 0 | 2.09 | 1.91 | 2.11 | 1.96 | 2.04 | 2.00 | 1.92 | 1.93 | 2.00 | 2.02 | 1.84 | 2.00 |
| 0 | 3 | 2.32 | 1.09 | 2.07 | 1.98 | 1.94 | 1.93 | 2.04 | 1.15 | 1.75 | 1.74 | 1.73 | 1.76 |
| 0 | 6 | 2.11 | 0.75 | 2.05 | 1.93 | 1.96 | 1.90 | 1.98 | 0.55 | 1.62 | 1.62 | 1.68 | 1.63 |
| 0 | 12 | 1.64 | 0.44 | 2.05 | 1.96 | 1.85 | 1.78 | 1.33 | 0.33 | 1.47 | 1.48 | 1.36 | 1.37 |
| 1 | 0 | 2.43 | 1.96 | 2.25 | 2.02 | 1.99 | 2.00 | 2.41 | 1.96 | 2.30 | 2.00 | 2.00 | 2.02 |
| 1 | 3 | 2.61 | 1.13 | 2.20 | 2.00 | 1.99 | 1.97 | 2.47 | 1.06 | 2.00 | 1.78 | 1.70 | 1.87 |
| 1 | 6 | 2.23 | 0.73 | 2.15 | 1.96 | 1.91 | 1.87 | 2.04 | 0.42 | 1.83 | 1.69 | 1.90 | 1.75 |
| 1 | 12 | 1.70 | 0.45 | 2.08 | 1.93 | 1.77 | 1.86 | 1.27 | 0.30 | 1.57 | 1.42 | 1.24 | 1.33 |

[a] SAA = the reagent combination of sulfanilic acid and *N*-(1-naphthyl)ethylenediamine. SAN = reagent combination of sulfanilamide and 1-naphthylamine.

curic chloride solution, 0.2 mL, was added to 2.0 mL slurry and mixed. The sample was then treated as described for direct analysis.

*AOAC method, 24.041-24.042* (4).—One mL samples were measured by positive displacement from a calibrated syringe into 100 mL volumetric flasks and diluted to ca 80 mL with water. Solutions were heated 2 h at 80°C, diluted to volume, and filtered through Whatman No. 2V paper. (Filter paper has been shown to be contaminated with nitrite (5) which interferes in the accuracy of the analysis. Sen and Donaldson (6) recommended discarding the first 20 mL filtrate but we found it necessary to wash the filters with 300–400 mL distilled water before washings were color-free with Griess reagents.) To 8 mL filtrate in 10 mL volumetric flask, 1 mL Griess reagent was added and the solution was diluted to volume. These solutions were then analyzed for nitrite.

*Griess reagent combinations.*—Two combinations used were 1.0 mM sulfanilic acid with 0.2 mM *N*-(1-naphthyl)-ethylenediamine (SAA), and 1.0 mM sulfanilamide with 0.2 mM 1-naphthylamine (SAN) prepared in 15% acetic acid. OSHA has classified 1-naphthylamine as a toxic and hazardous substance (CFR 29:1910.1004, 1979). The pigment concentrations in standard and sample solutions were determined from their absorbances. The absorptivities for standard solutions of sodium nitrite with NaCl or ascorbate added were $a_{mM} = 22.0$ at 540 nm for SAA and 42.5 at 525 nm for SAN.

## Results

### *Model System*

The results are shown in Table 1. In the absence of NaCl and ascorbate (first row), the concentration of nitrite measured was not affected by heat, sample preparation, or Griess reagent combination. The coefficient of variation of results for samples in the first row of Table 1 was 4%, which agrees well with the CV of 3% determined independently by replicate analyses of samples prepared by the 3 methods.

Two effects of ascorbate were observed on the nitrite measurement by direct analysis. The SAA reagent combination (column 3) increased apparent nitrite concentration with a maximum at 3 mM ascorbate, followed by a decrease at higher concentration. With the SAN reagents (column 4), a continuous decrease in apparent nitrite concentration with increasing levels of ascorbate was noted.

Both mercuric chloride and AOAC treatments eliminated the effects of ascorbate on the concentration of nitrite measured by both SAA and SAN reagent combinations in the unheated samples, although the AOAC procedure was not completely effective at the highest ascorbate concentration. When samples were heated, there was a loss of nitrite in the samples containing ascorbate, but the presence of residual ascorbate was indicated by the difference in nitrite concentrations measured by the 2 reagents in the direct reading samples. Again, AOAC and $HgCl_2$ treatments were effective in eliminating the ascorbate effect on pigment formation. There was also a chloride effect on the nitrite concentration measured by the 2 reagent combinations. Except in one set, the SAA values were always higher than the SAN values in the samples containing chloride, whether from NaCl or $HgCl_2$. Hildrum (3) has shown that chloride enhances pigment formation with sulfanilic acid, but in separate tests we found that chloride does

Table 2. Effects of NaCl, ascorbate, sample preparation methods, heat, and Griess reagents on measured nitrite (mM $NO_2^-$) in meat slurry [a] (pH 5.6; [$NO_2^-$] initial = 2.1 mM (145 ppm))

| | | Raw | | | | | | Cooked | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Direct | | $HgCl_2$ | | AOAC | | Direct | | $HgCl_2$ | | AOAC | |
| M NaCl | mM Asc. | SAA[a] | SAN | SAA | SAN | SAA | SAN | SAA | SAN | SAA | SAN | SAA | SAN |
| 0 | 0 | 2.23 | 2.15 | 1.79 | 1.70 | 2.07 | 1.90 | 1.85 | 1.87 | 1.64 | 1.37 | 2.01 | 1.91 |
| 0 | 3 | 2.79 | 1.64 | 1.76 | 1.65 | 2.21 | 1.83 | 2.09 | 1.38 | 1.36 | 1.27 | 2.00 | 1.76 |
| 0 | 6 | 2.63 | 1.27 | 1.72 | 1.63 | 2.62 | 1.61 | 1.98 | 1.01 | 1.33 | 1.20 | 2.04 | 1.54 |
| 0 | 12 | 2.29 | 0.81 | 1.77 | 1.67 | 2.70 | 1.28 | 1.58 | 0.66 | 1.22 | 1.13 | 1.91 | 1.16 |
| 0.5 | 0 | 2.33 | 2.40 | 2.29 | 2.07 | 1.82 | 1.73 | 2.09 | 1.79 | 2.02 | 1.76 | 1.98 | 1.91 |
| 0.5 | 3 | 2.72 | 1.61 | 2.07 | 1.97 | 1.86 | 1.63 | 1.84 | 1.19 | 1.60 | 1.39 | 1.45 | 1.43 |
| 0.5 | 6 | 2.72 | 1.24 | 2.00 | 1.90 | 2.14 | 1.56 | 1.58 | 0.87 | 1.45 | 1.28 | 1.45 | 1.24 |
| 0.5 | 12 | 2.33 | 0.81 | 2.00 | 1.93 | 2.37 | 1.26 | 1.35 | 0.53 | 1.31 | 1.18 | 1.45 | 0.93 |
| 1 | 0 | 2.45 | 1.91 | 2.16 | 1.97 | 2.01 | 1.85 | 2.20 | 1.83 | 2.03 | 1.71 | 1.84 | 1.76 |
| 1 | 3 | 2.42 | 1.62 | 2.08 | 1.91 | 2.08 | 1.78 | 1.98 | 1.17 | 1.71 | 1.30 | 1.54 | 1.37 |
| 1 | 6 | 2.44 | 1.20 | 2.03 | 1.84 | 2.50 | 1.29 | 1.49 | 0.71 | 1.36 | 1.07 | 1.30 | 1.02 |
| 1 | 12 | 1.84 | 0.81 | 2.18 | 1.87 | 2.20 | 1.27 | 0.93 | 0.39 | 1.03 | 0.86 | 1.09 | 0.73 |

[a] SAA = reagent combination of sulfanilic acid and *N*-(1-naphthyl)ethylenediamine. SAN = sulfanilamide and 1-naphthylamine.

not affect the amount of pigment formed from sulfanilamide. The difference is therefore due to the differential effect of chloride on the nitrosation of the 2 reagents.

### *Meat Slurry*

Heat caused a loss of nitrite in all but 3 of the meat slurry samples (Table 2). The loss in the samples with no added reductant (first row) was about 10% (significant at $P = 0.02$ from paired variate *t*-test) and was due to reaction with endogenous compounds in meat. An examination of all data, especially from the ascorbate-containing samples, shows that the measured nitrite was not uniform for any one of the factors studied, but was the result of interactions with the other experimental conditions. An analysis of variance (Table 3) showed that the variations could be accounted for by 4 three-factor interactions. Since the mean values for the 3-way combinations most clearly illustrate the more salient features of the corresponding 3-factor interactions, they are shown graphically in Figures 1–4.

*Heat Processing* × *Salt* × *Ascorbate.*—The mean values ($n = 6$) for the first combination in Table 3 are shown in Figure 1. Measured nitrite decreases in both raw and cooked samples with both salt and ascorbate. Because the raw samples were analyzed for nitrite immediately after being prepared, there was little time for nitrite to react with tissue components or added ascorbate, and the observed regression is due to ascorbate interference in the Griess reaction. Although chloride has no effect on the measured concentration of nitrite in the raw samples, the loss of nitrite in the cooked samples due to ascorbate was enhanced by increasing chloride concentration. Assuming nitrite loss on heating to be

Table 3. Significant 3-factor interactions from analysis of variance of data of Table 2

| Interaction | *F* ratio | Significance, *p* |
|---|---|---|
| Processing × salt × ascorbate | 3.42 | 0.033 |
| Processing × preparation × reagent | 20.3 | 0.0001 |
| Processing × reagent × ascorbate | 10.9 | 0.0010 |
| Prepartion × reagent × ascorbate | 22.9 | 0.0001 |

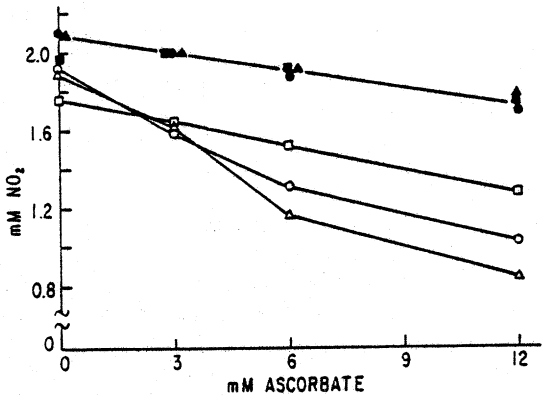


Figure 1. Mean values ($n = 6$) illustrating 3-factor interaction of heat, salt, and ascorbate concentration effects on measured nitrite in pork slurry. □, 0 salt; ○, 0.5M NaCl; △, 1.0M NaCl. Solid symbols, unheated; open symbols, heated.

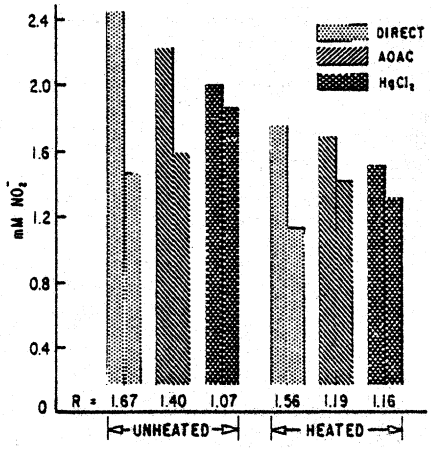


**Figure 2. Mean values ($n$ = 12) illustrating 3-factor interaction of heat, preparation method, and reagent on measured nitrite. First bar in pair SAA, second SAN. Figure below reagent pair is ratio of SAA to SAN values.**

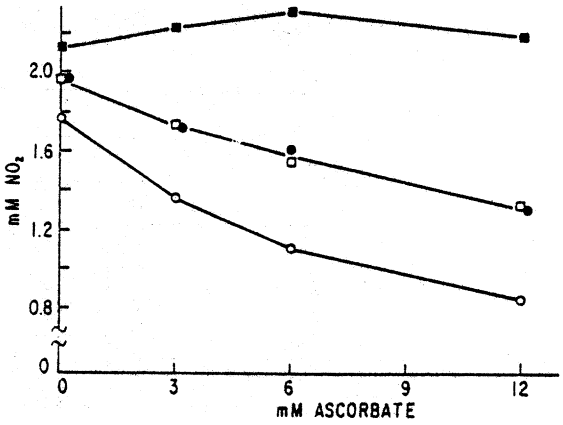


**Figure 3. Mean values ($n$ = 9) illustrating 3-factor interaction of heat, reagent, and ascorbate concentration on measured nitrite. □, SAA; ○, SAN. Solid symbols, unheated; open symbols, heated.**

related to the reactivity of the nitrosating species, this chloride enhancement may be attributed to the formation of nitrosylchloride (NOCl), a more reactive nitrosating species than $N_2O_3$, the species formed from nitrite alone (11). On the basis of the observation that loss of nitrite was not proportional to chloride level in the model system, it may be attributable to NOCl being more reactive than $N_2O_3$ with meat tissue components.

*Heat Processing* × *Preparation* × *Reagent.*—The difference in measured nitrite by the 2 colorimetric reagents (Table 2) was a function of both processing and method of preparation (second interaction, Table 3). The mean values ($n$ = 12) are plotted in Figure 2. In addition to the decrease in measured nitrite after heating, the difference between the 2 colorimetric reagents was less in the cooked than in the raw samples for both the direct and AOAC preparation methods. As was observed in the model system, the reagent difference is due to residual ascorbate, which was only partially destroyed in the meat slurries by the heating process. The difference was minimal in the $HgCl_2$ samples because ascorbate was removed by precipitation with mercuric ion, but the chloride effect was still present.

*Heat Processing* × *Reagent* × *Ascorbate.*—Table 2 shows that, although the nitrite concentrations in the unheated samples measured by SAA were relatively constant, there was a regression with ascorbate in the heated/SAA samples and in all the SAN samples. This 3-factor interaction was significant ($P$ = 0.0001); the mean values ($n$ = 9) are shown in Figure 3. In the unheated samples the ascorbate enhancement of pigment formation from the SAA reagent is observed, as well as the decrease in the pigment formed from the SAN reagent. In the heated samples, the SAA values were uniformly higher than the SAN values, due to both the ascorbate and chloride enhancement of SAA pigment formation, but there was a significant regression of measured nitrite with ascorbate in the SAA samples. The coincidence of the SAA/heated and SAN/unheated data is happenstance and has no significance.

*Preparation* × *Reagent* × *Ascorbate.*—The fourth 3-factor interaction in Table 3 is the most relevant to the intent of this study in that it shows the effect of the sample preparation techniques on the ascorbate interaction with the 2 colorimetric reagents. The mean values ($n$ = 6) for this interaction are shown in Figure 4. The regressions of measured nitrite with ascorbate concentration are not linear because nitrite loss is a function of the square root of the ascorbate concentration (12, 13). The differential effects of both chloride and ascorbate on pigment production are observed in the curves of the direct and AOAC procedures, but only the differential chloride effect on pigment production from the 2 reagent combinations in the $HgCl_2$ procedure. The initial values differ because of the chloride effect, but the direct and AOAC curves diverge with increasing ascorbate, indicating residual ascorbate. Because the AOAC procedure was effective in removing ascorbate in the model systems, it is evident that the ascorbate in the slurries was protected against

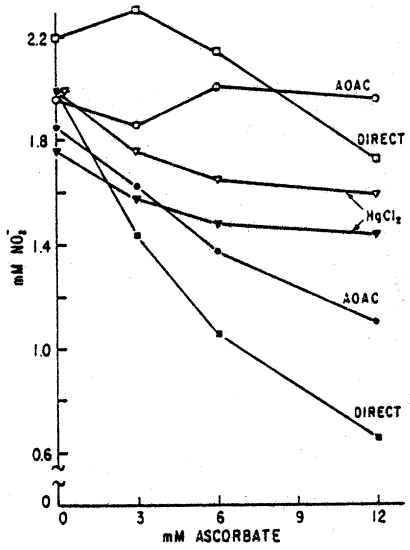


**Figure 4. Mean values ($n$ = 6) illustrating 3-factor interaction of preparation method, colorimetric reagent, and ascorbate. □, Direct reading; ○, AOAC; △, $HgCl_2$. Solid symbols, SAN; open symbols, SAA.**

destruction during the heating period of the AOAC procedure. Meat contains residual reductants produced during glycolysis, which apparently protect the ascorbate against oxidation at higher temperatures.

The presence of residual ascorbate in the samples leads to an artifact in the measurement of nitrite. In Figure 4, the mean value curve for the AOAC/SAA combination shows no apparent loss in nitrite with increasing ascorbate. The experimental data in Table 2 also show several examples of this same lack of dependence, e.g., the AOAC/SAA values for the cooked, no-salt samples. This phenomenon, a result of the combined effects of ascorbate on nitrite loss and enhancement/decrease of pigment formation, explains an apparent disagreement in the literature. Sen and McPherson (14) reported a 1.5% loss of nitrite with 2 μg/mL ascorbate, while Adriaanse and Robbers (1) reported a 49% loss at the same ascorbate concentration. This difference in percent loss may be explained by the use of the sulfanilic acid/*N*-(1-naphthyl)-ethylenediamine combination by Sen and McPherson and the use of sulfanilamide and 1,7-Cleve's acid (8-amino-2-naphthalenesulfonic acid) by Adriaanse and Robbers.

## Conclusions

The results of this study show that the amount of nitrite measured in a given sample is a function of its prior treatment, that is, whether heat-processed or not, its composition, the way it is prepared for analysis, and the reagent used for nitrite measurement, all of which factors interact. Under these conditions it is difficult to establish a true or correct value for the amount of nitrite in the sample, unless some criteria are available to show whether a specific interference or interaction has been eliminated. We have established such a criterion in the differential effect of residual ascorbate on the production of pigment from the 2 reagent combinations, SAA/NED and SAN/1-NA. Application of this criterion to our data indicates that the use of mercuric chloride is superior to the AOAC procedure for removing ascorbate from meat systems, but we do not specifically recommend the use of $HgCl_2$ as there may be other procedures as effective and more environmentally acceptable.

A second criterion is that the amount of pigment produced by a given set of colorimetric reagents should be the same in both standards and substrates. This can be achieved either through appropriate sample preparation to remove interfering compounds or by selection of appropriate reagents. The second alternative is superior to the first because it requires less handling of the sample. By this reasoning, SAN is preferred to SAA because the amount of pigment produced from the former does not depend on the salt concentration. Most official methods use SAN (4, 7, 8), but SAA has been used in recent studies (3, 6).

We recommend the use of the 2 reagent combinations, sulfanilic acid/*N*-(1-naphthyl)-ethylenediamine and sulfanilamide/1-naphthylamine, as a quick method for detecting residual ascorbate while determining nitrite. We also recommend the use of sulfanilamide as a Griess colorimetric reagent because pigment production from it is insensitive to chloride concentrations.

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